RESPONSE TO OFFICE ACTION

A. Status of the Claims

The Action notes that all of the claims have been examined in the instant case upon reconsideration of the Restriction Requirement. Applicants thank the Examiner for reconsidering the restriction. Claims 1-89 were therefore examined. Claims 2 and 3 have been cancelled herein and claims 1, 13, 17, 38, 57, and 74 amended. Claims 1 and 4-89 are currently pending and are presented for reconsideration.

B. Rejection of Claims Under the Written Description Requirement of 35 U.S.C. §112, First Paragraph

The Action rejects claims 1-89 for failing to comply with the written description requirement of 35 U.S.C. § 112, first paragraph. Specifically, the Action alleges that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey possession of the invention at the time the application was filed. Applicants respectfully traverse as set forth below.

1. Written Description Must Be Analyzed with Respect to the Claimed Invention

It is initially noted that the rejection appears to require Applicants to show how or why the claimed promoter sequences function. In particular, the Examiner suggests that Applicants must show which structural features are necessary for the function of the glutamine synthetase GS_{1-2} promoter. However, what is relevant under 35 U.S.C. § 112, first paragraph is that Applicants were in possession of the ***claimed invention***; and not unclaimed features envisioned in the Action. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991).

The claims are not directed to particular functional elements. For example, claim 1 of the application currently reads as follows:

> 1. (Currently amended) An isolated nucleic acid sequence comprising a cytoplasmic glutamine synthetase GS1-2 promoter, wherein the cytoplasmic glutamine synthetase GS1-2 promoter comprises:
> (a) a nucleic acid sequence of SEQ ID NO:18 or a fragment thereof, having promoter activity, wherein the fragment comprises from 400 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18; or
> (b) a nucleic acid sequence that hybridizes to the nucleic acid sequence of SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes.

Thus what is relevant for purposes of written description is that Applicants teach at least 400 nucleotides of SEQ ID NO:18. Applicants have done so by providing the nucleic acid sequence of SEQ ID NO:18 in the sequence listing and in FIG. 2, both of which were provided with the specification as filed. Applicants do not lack a written description for what is expressly set forth in the application. While the claims encompass nucleic acid sequences that hybridizes to SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30, these conditions are stringent and thus define a subset of sequences fully described by SEQ ID NO:18. It is well settled that the Applicants need not provide an *ipsis verbis* description for the claimed invention. *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) (stating that the written description requirement does not require an applicant to "describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed" (citations omitted)). Here, the entire scope of claimed subject matter is supported by the literal description in the sequence listing. *The Regents of The University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1568; 43 USPQ2d 1398, 1406 (Fed. Cir. 1997) (noting that a name alone does not satisfy the written description requirement where "it does not define any structural features commonly possessed by members

of the genus that distinguish them from others. One skilled in the art therefore cannot, *as one can do with a fully described genus, visualize or recognize the identity of the members of the genus*" (emphasis added)). All of the claimed subject matter has therefore been fully described pursuant to 35 U.S.C. § 112, first paragraph.

2. The Holding of *Fiers* is Inapplicable to the Current Claims

In the instant case, the independent claims recite a minimum of 400 contiguous nucleotides of SEQ ID NO:18. Nonetheless, the Action relies on *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993) for the proposition that the instant claims lack written description. However, *Fiers* is not applicable to the instant situation and involves a fact pattern distinct from the current situation. *Fiers* involved a three-way interference proceeding over DNA coding for a human fibroblast interferon polypeptide. With respect to the written description requirement, the three parties were entitled to the priority date of their foreign applications only if those applications disclosed the nucleotide sequence of the claimed interferon gene. One of the parties' foreign applications could not be used to establish priority because it merely disclosed a method that might be used to obtain mRNA coding for the claimed interferon gene.

Here, however, the Applicant's specification has provided the claimed nucleic acid sequences by way of SEQ ID NO:18. Applicants have further provided a written description of the subfragments of SEQ ID NO:18. For example, at page 7, lines 1-7, subfragments of SEQ ID NO:18 are described comprising at least about 135, 250, 400, 750, 1000, 1500, 1750, 2000, 2250, and 2500 contiguous nucleotides up to the full 2547 nucleotides of SEQ ID NO:18. These sequences all have literal support in the sequence listing. In view of the nucleic acid sequence of

SEQ ID NO:18 provided in the sequence listing and in FIG. 2, there is no basis to allege that the Applicants did not clearly convey possession of the claimed invention.

In view of the foregoing, Applicants assert that the specification satisfies the written description requirement. Removal of the rejection under 35 U.S.C. § 112, first paragraph is thus respectfully requested.

C. Rejection of Claims Under the Enablement Requirement of 35 U.S.C. §112, First Paragraph

The Action rejects claims 1-89 as not being enabled by the specification. In particular, the Action alleges that the specification is enabling only for the entire 2547 nucleotide sequence of SEQ ID NO:18.

1. The Enablement Requirement Must Be Applied With Respect to the Claimed Invention

The Action alleges that the specification does not disclose certain structural and functional information regarding which fragment derived from or hybridizing to SEQ ID NO:18 would be likely to have promoter function. However, Applicants note that which structural or functional elements are present is irrelevant. Applicants need not describe why or how the invention works. All that is required under 35 U.S.C. §112, first paragraph, is that the specification teaches one reasonably skilled in the art how to make and use the claimed invention without undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). That is, Applicants must only enable what is claimed. See *Durel Corp. v. Osram Sylvania Inc.*, 256 F.3d 1298, 1306-07 (Fed. Cir. 2001). Here, the claimed invention represents contiguous sequences of 400 nucleotides or more of SEQ ID NO:18, the use thereof and

compositions produced therefrom. As described below, the specification has fully enabled this subject matter.

2. Applicants Have Affirmatively Demonstrated Compliance With the Enablement Requirement

The current claims recite nucleic acids comprising at least 400 contiguous nucleotides of SEQ ID NO:18. Provided in the Sequence Listing of the specification is the nucleic acid sequence of SEQ ID NO:18. This is more than adequate to fully enable one of skill in the art to prepare nucleic acid sequences of at least 400 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18. The Action, however, suggests that one of skill in the art would be without guidance in obtaining the claimed fragments of this sequence because the specification does not provide the "minimal functional promoter regions derived from SEQ ID NO:18." However, the structural information of this subject matter is the sequence itself, which is given in SEQ ID NO:18.

The Action further ignores extensive teaching in the specification that goes well beyond what is required under the first paragraph of 35 U.S.C. § 112. For example, provided in the specification from page 16, line 21 to page 20, line 7 is detailed teaching regarding the preparation of derivatives of the full length promoter sequence. Described from page 20, line 11 to page 31, line 12 of the specification are plant transformation constructs comprising the promoter compositions, as well as elements for inclusion in these constructs. Described in detail from page 74, line 24 to page 81, line 15 are numerous methods that are known to those of skill in the art for transforming plants including, for example, direct delivery of DNA by PEG-mediated transformation of protoplasts, desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by *Agrobacterium*-mediated

transformation and acceleration of DNA coated particles. Described from line 19, page 81 to line 21, page 89 are methods for culturing recipient cells for transformation. Described from page 89, line 25 to page 99, line 14 are methods for the production and characterization of stably transformed plants, including methods for assaying for transgene expression.

In the working examples, Example 1 at page 108-112 describes the isolation of the maize pedicel-specific glutamine synthetase GS_{1-2} promoter from genomic DNA of *Zea mays* variety 01IBH2 using an inverse PCR strategy with primers designed from the glutamine synthetase GS_{1-2} disclosed by Li *et al.*, (1993; accession number X65927). A 2.7 Kb fragment containing the glutamine synthetase GS_{1-2} promoter region was identified.

Example 2 at page 112 describes the construction of transformation constructs comprising the glutamine synthetase GS_{1-2} promoter. In Example 4, the bombardment of H99 immature embryos with constructs comprising the glutamine synthetase GS_{1-2} promoter is described. In particular, the *uidA* (GUS) reporter gene was fused to the glutamine synthetase GS_{1-2} promoter for evaluation of expression stable transformation experiments. Example 5 describes the regeneration of transgenic plants from transformed H99 maize cells.

Still further, Example 6 describes analysis of glutamine synthetase GS_{1-2} promoter expression in transgenic maize. Small regenerated plants from a number of independent transformation events were assayed for GUS activity by histochemical staining and were positive for *uidA* reporter gene expression. The R_o plants were crossed to a proprietary inbred line (H99) or were self pollinated and resultant R_1 plant progeny, as well as progeny of subsequent generations, were analyzed for GUS expression. GUS expression, as directed by the glutamine synthetase GS_{1-2} promoter, was observed in the cob vasculature, pedicel, basal conducting cells, and silk scar (Table 8).

Further, other detailed teaching for the transformation and assaying of plants with selected nucleic acids is contained in the remaining examples. In particular, Example 3 teaches the preparation of microprojectiles in significant detail, while Example 7 teaches *Agrobacterium tumefaciens*-mediated transformation.

Further detailed teaching of the methods for producing and screening promoter constructs is described in section VIII of the Detailed Description of the Invention, entitled "Production and Characterization of Stably Transformed Plants." For example, this section describes (1) selection of transformants, (2) regeneration of transgenic plants and seed production, and (3) genetic characterization to confirm transgenic plants, including confirmation of the presence and expression of transgenic sequences. Screening techniques for quantifying expression are described that allow the quantitation and detection of RNA produced from introduced genes, as well as by the expression of screenable marker genes as is described in the working examples. The foregoing teachings in Applicants' specification are more than adequate to enable the full scope of the invention and cannot properly be ignored. *In re Wands*, 858 F.2d at 737.

It would be a straightforward matter for one of skill in the art to identify subfragments of SEQ ID NO:18 having promoter activity, especially given the detailed teachings in the specification. While Applicants acknowledge this would require some routine screening, "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands*, 858 F.2d at 737. Furthermore, some amount of experimentation is permissible, especially when the specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Id.* (quoting *Ex parte Jackson*, 217 USPQ2d 804, 807 (Bd. App. 1982)). The detailed teaching in the specification has

provided all of the methodology necessary for creating and screening the subject subfragments for promoter activity.

Given the fact that SEQ ID NO:18 is only 2547 nucleic acids long, nothing could be further from the truth than to suggest that it would require undue experimentation to create subfragments of this sequence and screen them for activity. This is underscored by the fact that the working examples provide exactly the methodology that one could use to screen subfragments for activity, namely inserting promoter fragments of SEQ ID NO:18 into a transformation construct, as described in Example 2 for the full length sequence; transforming recipient cells with the constructs, as described in Example 4; and screening the subsequently derived transgenic plants for activity, as described in Examples 5 and 6 of the specification. These teaching fully demonstrate the enablement of the claims.

It must further be noted that it is well known that full length promoter fragments can be substantially deleted and mutated while still retaining promoter activity. For example, Cho and Cosgrove (2002) (*Plant Cell*, 14, 3237–3253) showed that more than 990 base pairs of an approximately 1428 bp plant promoter sequence designated AtEXP7 could be deleted without significantly effecting promoter activity and even larger deletions could be made while maintaining a reduced promoter activity. See **Exhibit A**, p. 3244, 2nd col. and FIG. 8. It was also shown that a deletion of approximately 775 bp could be made from a 1058 bp plant promoter designated AtEXP18 without significantly reducing promoter activity. See FIG. 10. The authors further showed that numerous substitution mutations could be made in a fragment of AtEXP7 retaining full activity while retaining promoter activity and in some cases increasing activity. See. FIG. 9 and p. 3245, 2nd col. These studies therefore show that fragments of full length promoter sequences can routinely be made that retain promoter activity.

3. The Action Has Failed to Establish a *Prima Facie* Case of Lack of Enablement

The Action has provided no basis to doubt the enablement of the instant claims. Rather, the Action merely states that the specification does not provide sufficient structural and functional information regarding the claimed sequences for one of skill in the art to predict which species of SEQ ID NO:18 would have promoter activity without any objective basis for so concluding. However, it is the PTO that bears the burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by a claim is not adequately enabled by the description of the invention in the specification. *In re Wright*, 9 U.S.P.Q.2d 1510, 1512-1513 (Fed. Cir. 1993) (citing *In re Marzocchi*, 169 U.S.P.Q. 367, 369-70 (CCPA 1971)). Further, the evidence presented above demonstrates that one of skill in the art in view of the teaching in the specification could readily have made the claimed sequences without undue experimentation. A statement doubting the enablement of an Applicants' claims without providing an objective basis does not meet this standard, "[o]therwise, there would be no need for the Appellant to go to the trouble and expense of supporting his presumptively accurate disclosure." *In re Marzocchi*, 169 U.S.P.Q. at 370. Thus, without more, the rejection must fail.

It is finally noted that the legal standard for enablement does not require that Applicants demonstrate enablement for all possible claimed iterations. Enablement must bear only a reasonable relationship to the scope of the claims. *In re Fisher*, 166 U.S.P.Q. 18, 24 (CCPA 1970). For example, a patent applicant is not required to "predict every possible variation, improvement or commercial embodiment of his invention." *United States Steel Corp. v. Phillips Petroleum Co.*, 673 F. Supp. 1278, 1292 (D. Del. 1987), *aff'd,* 865 F.2d 1247, 1250 (Fed. Cir. 1989) (specifically quoting this statement). This is echoed in the MPEP: "[a]s long as the

specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied." MPEP 2164.01(b) (citing *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (CCPA 1970)).

In view of the foregoing, Applicants respectfully submit that the full scope of the claims has been enabled. Removal of the rejection under 35 U.S.C. §112, first paragraph for lack of enablement is thus respectfully requested.

D. Rejection of Claims Under 35 U.S.C. § 112, Second Paragraph

The Action rejects claims 13-14 and 17-89 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In particular, the Action asserts that the limitations "the enhancer comprises an intron," "the maize," and "the selected heterologous coding region" appearing in claims 13, 17, 18, 20, 38, 57, and 74 lack a sufficient antecedent basis.

In response, Applicants note the claims have been amended and that the rejections are now moot. The amendments do not narrow the claims and Applicants do not intend to disclaim any subject matter through the amendment.

In view of the foregoing, removal of the rejection is respectfully requested.

E. Rejection of Claims Under 35 U.S.C. § 102(e)

The Action rejects claims 1, 11-13, 15, 17-18, 20, 22, 24-25, 28-30, 34-37, 57-66, 69-70, and 72 under 25 U.S.C. § 102(e) as allegedly anticipated by Muhitch (U.S. Patent Pub. No.

US20040148651). The Action also rejects claims 1-2 and 11 as allegedly anticipated by La Rosa et al. (U.S. Patent Pub. No. US20040214272A1).

In response, Applicants note claim 1 has been amended. Claim 1 currently recites:

> 1. An isolated nucleic acid sequence comprising a cytoplasmic glutamine synthetase GS1-2 promoter, wherein the cytoplasmic glutamine synthetase GS1-2 promoter comprises:
> (a) a nucleic acid sequence of SEQ ID NO:18 or a fragment thereof, having promoter activity, wherein the fragment comprises from 400 to 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18; or
> (b) a nucleic acid sequence that hybridizes to the nucleic acid sequence of SEQ ID NO:18 under wash conditions of 2X SCP, 1% SDS at 65°C for 30 minutes.

In light of the amendments, neither Muhitch nor La Rosa *et al.* teach all of the limitations of claim 1 and specifically do not teach the nucleic acids recited in (a) and (b) above. Therefore, it is believed that the rejections are moot in light of the amendments, and the Applicants respectfully request removal of the rejections.

F. Rejection of Claims Under 35 U.S.C. § 103

The Action rejects claims 1 and 11-89 under 35 U.S.C. § 103(a) as obvious over Muhitch in combination with a number of other prior art references. In response, Applicants note that none of the references teach the claimed nucleic acids as shown in claim 1 and explained above. As all elements of the claims are not found in the prior art, the claims cannot be deemed obvious. Removal of the rejection is thus respectfully requested.

CONCLUSION

In view of the foregoing, Applicants respectfully request favorable consideration of this case and withdrawal of the Restriction Requirement.

The Examiner is invited to contact the undersigned attorney at (512) 536-3085 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,

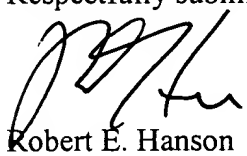

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EXHIBIT A

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Regulation of Root Hair Initiation and Expansin Gene Expression in Arabidopsis[W]

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The expression of two Arabidopsis expansin genes (*AtEXP7* and *AtEXP18*) is tightly linked to root hair initiation; thus, the regulation of these genes was studied to elucidate how developmental, hormonal, and environmental factors orchestrate root hair formation. Exogenous ethylene and auxin, as well as separation of the root from the medium, stimulated root hair formation and the expression of these expansin genes. The effects of exogenous auxin and root separation on root hair formation required the ethylene signaling pathway. By contrast, blocking the endogenous ethylene pathway, either by genetic mutations or by a chemical inhibitor, did not affect normal root hair formation and expansin gene expression. These results indicate that the normal developmental pathway for root hair formation (i.e., not induced by external stimuli) is independent of the ethylene pathway. Promoter analyses of the expansin genes show that the same promoter elements that determine cell specificity also determine inducibility by ethylene, auxin, and root separation. Our study suggests that two distinctive signaling pathways, one developmental and the other environmental/hormonal, converge to modulate the initiation of the root hair and the expression of its specific expansin gene set.

INTRODUCTION

Root hairs are polarized outgrowths of root epidermal cells. In Arabidopsis, root hairs normally arise from epidermal cells that contact two underlying cortical cells (the so-called H position), whereas epidermal cells overlying a single cortical cell (in the N position) develop into nonhair cells (Dolan et al., 1993; Galway et al., 1994). This position-dependent hair cell differentiation thus results in a striped pattern of hair cell files along the long axis of the root, which is found in members of Brassicaceae and in a few species of other families (Cormack, 1947; Dolan and Costa, 2001). Root hair development in Arabidopsis can be divided into three phases: cell specification, initiation, and elongation. Cell specification refers to the fate determination of epidermal cells into hair cells and nonhair cells, depending on position. Initiation refers to the formation of a protrusion or bulge at the site of hair outgrowth. Elongation refers to the process of sustained tip growth that normally follows initiation. Numerous experimental observations indicate that these three phases involve different cellular and genetic processes (for reviews, see Schiefelbein, 2000; Foreman and Dolan, 2001).

Several genes that control root epidermal cell specification have been identified. Loss-of-function mutations in TTG (TRANSPARENT TESTA/GLABROUS) or GL2 (GLABRA2) result in root hairs in both H and N positions (Galway et al., 1994; Masucci et al., 1996), indicating that TTG (a protein with WD40 repeats) and GL2 (a homeodomain transcription factor) function as negative regulators of the differentiation of nonhair cells to hair cells. Mutations in another MYB transcription factor, WER (WEREWOLF), also generate root hairs in almost every root epidermal cell, because WER positively regulates GL2 expression (Lee and Schiefelbein, 1999). On the other hand, *cpc* (*caprice*) mutants have only a few root hairs, indicating that CPC, a MYB-like protein, functions as a positive regulator for root hair cell differentiation (Wada et al., 1997). A recent study demonstrated the interactions among these regulatory genes (Lee and Schiefelbein, 2002). In the N position, WER positively regulates the expression of CPC and GL2. CPC (or its downstream signal) appears to move to cells in the H position and inhibits the expression of WER, CPC, and GL2, which leads the cell to initiate hair formation.

Root hair initiation, which is genetically downstream of GL2 (Masucci and Schiefelbein, 1996), is regulated by another set of genes and is sensitive to hormonal and environmental factors (Schiefelbein, 2000). The auxin-resistant mutant (*axr2*) develops few root hair bulges (Wilson et al., 1990), and the defect of root hair initiation in root hair defective (*rhd6*) can be reversed by treatment with auxin or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid

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[W] Online version contains Web-only data.

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(ACC) (Masucci and Schiefelbein, 1994, 1996). ACC treatment of wild-type plants induces root hairs in the N position, as do the constitutively ethylene-responsive *ctr1* and ethylene-overproducing *eto* mutants (Dolan et al., 1994; Masucci and Schiefelbein, 1996; Cao et al., 1999). ACC has been suggested as a factor that determines the developmental fate of cells in the H position (Tanimoto et al., 1995). Also implicating ethylene involvement in root hair initiation, the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) and silver ion (an inhibitor of ethylene perception) have been found to inhibit root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995). However, the role of ethylene in root hair formation is questioned because the ethylene-insensitive mutants *etr1* and *ein2* maintained normal root hair numbers (Masucci and Schiefelbein, 1996). Additionally, environmental factors such as nutrients (Peterson and Stevens, 2000), light, and separation of the root from the agar medium (Okada and Shimura, 1994) also affect root hair development. It has been suggested that hormones and environmental factors affect root hair initiation through a pathway distinctive from the normal development-associated pathway (Okada and Shimura, 1994; Schiefelbein, 2000), but experimental confirmation for this is needed.

Elongation of the root hair is achieved by tip growth (Schiefelbein, 2000). Hair elongation likely is governed by genetic components distinct from those that govern hair initiation, but root hair elongation is influenced by auxin, ethylene, and environmental factors as well (Okada and Shimura, 1994; Pitts et al., 1998; Schiefelbein, 2000).

Spatial regulation of cell wall expansion is critical for cell morphogenesis in plants (Fowler and Quatrano, 1997). Thus, outgrowth of the root hair from the epidermal cell is expected to accompany localized cell wall loosening at the correct position. Bibikova et al. (1998) demonstrated localized wall acidification at the site of root hair initiation. This acidification could activate expansins. Expansins are cell wall–loosening proteins capable of mediating cell wall extension in acidic conditions without hydrolytic breakage of major structural components of the cell wall (McQueen-Mason et al., 1992; for recent reviews, see Cosgrove, 2000; Lee et al., 2001). Expansin genes are found throughout the entire plant kingdom (Cosgrove, 1999; Li et al., 2002), and their pattern of expression indicates that they are related closely to cell growth and tissue differentiation (for review, see Cho, 2001). Alteration of endogenous expansin gene expression modulates leaf growth and pedicel abscission in Arabidopsis (Cho and Cosgrove, 2000) and leaf morphology and phylotaxy in tobacco (Pien et al., 2001). Two families of expansins are recognized at present (Cosgrove, 2000), α- and β-expansins, and Arabidopsis has 26 α- and 5 β-expansin genes (see http://www.bio.psu.edu/expansins). In the course of analyzing the expression of these genes in Arabidopsis, two α-expansin genes, *AtEXP7* and *AtEXP18*, were found to be expressed specifically in root hair cells (D.M. Durachko and D.J. Cosgrove, unpublished data).

In this study, we examined in detail the expression patterns of these two root hair–specific expansin genes in various root hair mutants as well as under hormonal (auxin and ethylene) and environmental (separation of the root from the medium) treatments. In particular, the role of endogenous ethylene in root hair development was studied closely. Promoter analyses of the two expansin genes, in conjunction with the effect of root hair–inducing factors, also were conducted to elucidate the regulation of expression of these root hair–specific genes. Our results show that the expression of these expansin genes is linked tightly to root hair initiation and subsequent elongation. Moreover, we find that, although ethylene mediates the effects of auxin and root separation on root hair initiation, it is not essential for the normal (or default) development of root hairs in wild-type plants. These results alter current views of ethylene involvement in root hair development.

RESULTS

Root Hair Cell–Specific Expression of *AtEXP7* and *AtEXP18*

RNA gel blot and promoter-reporter gene expression analyses were performed to investigate the organ- and tissue-specific expression patterns of *AtEXP7* and *AtEXP18*. The transcripts of both expansin genes were found in the root but were undetectable in other major plant organs (Figure 1). Wild-type plants harboring the *AtEXP7* promoter::β-glucuronidase (*GUS*) or *AtEXP7* promoter::green fluorescent protein (*GFP*) construct showed staining (or fluorescence) solely

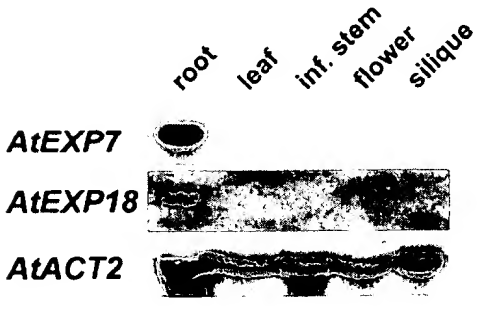

Figure 1. Expression of *AtEXP7* and *AtEXP18* in Different Tissues.

Total RNA was isolated from seedling roots, young leaves, growing inflorescence (inf.) stems, whole floral organs, and young green siliques of Columbia wild-type Arabidopsis plants. Twenty micrograms of total RNA was analyzed per lane. The transcript levels of Arabidopsis actin2 (*AtACT2*) served as a loading control.

in root hair cell files (Figures 2A to 2D). No reporter gene expression was found in other cell types of the root or other organs except a weak expression in the inner layer of the seed coat. Different ecotypes, Columbia and Wassilewskija, showed the same reporter gene expression pattern. The expression of *AtEXP7* occurred approximately one cell before the root hair bulges appeared (Figure 2B), indicating the gene's close temporal expression with the hair initiation process. Plants harboring the *AtEXP18* promoter::reporter construct also showed the same expression pattern as plants with the *AtEXP7* promoter::reporter construct (data not shown). However, the level of *AtEXP18* expression was lower than that of *AtEXP7*. Promoter analyses, as described below, showed that the average promoter activity of *AtEXP18* was ~60% of *AtEXP7* promoter activity. In this study, the expression pattern of *AtEXP7* is described in greater detail, but the results also hold for *AtEXP18*.

The AtEXP7 protein expression pattern also was examined by expressing the AtEXP7-GFP fusion protein driven by the *AtEXP7* promoter. The cell-type specificity and the timing of protein expression were almost identical with the expression pattern of the reporter gene alone (Figures 2E to 2G). The fluorescence from the fusion protein was highest in regions of root hair initiation and elongation. Although the AtEXP7-GFP fusion protein tended to localize more at the emerging root hair tip and to distribute peripherally in the root hair cell (Figures 2E to 2G), it was detected predominantly inside the plasma membrane upon plasmolysis (data not shown). This finding indicates that the fusion protein was not secreted to the cell wall.

We have searched for mutants defective in *AtEXP7* or *AtEXP18*. An Arabidopsis line that includes a T-DNA insertion in the second intron of *AtEXP7* was identified, but the homozygous line still expressed transcripts of the correct size, albeit at a lower level than in the wild type. This line did not show obvious alterations in the root hair, most likely as a result of the leakiness of the mutation and functional redundancy by *AtEXP18* and perhaps other expansin genes.

Effect of Root Hair–Regulating Factors on the Expression of Root Hair Expansin Genes

Root hair formation in Arabidopsis is regulated by developmental regulators, hormones, and environmental factors. Because *AtEXP7* is a root hair–specific gene and is thought to function in root hair formation, we investigated whether *AtEXP7* expression is modulated by various root hair–regulating factors. For this purpose, the *AtEXP7* promoter::*GUS* reporter construct was introduced into root hair mutants, and the reporter gene expression pattern was monitored.

In *ttg* and *gl2* mutants, which have hairs in both the H and N positions, *AtEXP7* promoter::*GUS* was expressed in both positions (Figures 2H and 2I), suggesting that TTG and GL2 negatively regulate the expression of *AtEXP7*, just as they negatively regulate root hair formation in the N position of the wild-type plant (Galway et al., 1994; Masucci et al., 1996).

The *axr2* mutant is defective in hair elongation and partially in hair initiation; thus, it produces few root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Figure 2J). The spatial pattern of *AtEXP7* promoter::*GUS* expression was not changed in this mutant (Figure 2J) compared with that in wild-type plants. However, the expression level of *AtEXP7* was much lower in the mutant than in the wild type (Figure 3). Because auxin positively regulates root hair formation and *AtEXP7* expression (see below), AXR2 likely downregulates the expression of *AtEXP7* and partially inhibits root hair formation.

The mutant *rhd6* also is defective in root hair initiation, but unlike *axr2*, it develops almost no root hair bulges (Masucci and Schiefelbein, 1994, 1996) (Figure 2K). *AtEXP7* expression in *rhd6* was blocked almost completely, as shown by GUS expression and by transcript analysis (Figures 2K and 3). In *rhd6*, treatment with the ethylene precursor ACC or auxin, or separation of the root from the agar medium induced normal root hair formation (Masucci and Schiefelbein, 1994, 1996) (Table 1). In agreement with their effects on root hair formation, all of these treatments induced *AtEXP7* expression in *rhd6* roots (Figures 2L to 2N and 3). *AtEXP18* expression in *rhd6*, as described below, also was inducible by these treatments. These results indicate that RHD6 is a positive regulator of *AtEXP7* and *AtEXP18* expression.

Exogenous Ethylene Is a Positive Effector for the Expression of Root Hair Expansin Genes in Concert with Root Hair Formation

Because ethylene is a positive effector of root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995), we investigated whether ethylene coordinately regulates the expression of root hair expansin genes with root hair formation. The ethylene precursor ACC (5 μM) induced root hair formation and *AtEXP7* expression in the N position of the wild-type root (Figure 2O). Mutation in CTR1, which showed constitutive ethylene effects and thus induced the formation of root hairs in the N position (Table 1), likewise activated *AtEXP7* expression in root hairs in the N position (Figure 2R) and increased the transcript level by 36% relative to that of the wild type (Figure 3). Compared with the wild type, the root hair–defective *rhd6* mutant had only ~10% of the *AtEXP7* transcript (Figure 3), which could derive from the occasional root hairs in the *rhd6* root. Treatment of the mutant with 5 μM ACC restored 78% of the transcript level and 74% of the root hair number (Table 1). Ethylene gas (1 μL/L) treatment also induced a similar level of root hairs in the *rhd6* root, as did 5 μM ACC (data not shown), and the effect of exogenous ethylene or ACC could be blocked completely by 1-methylcyclopropene (1-MCP), the competitive inhibitor of ethylene binding to the

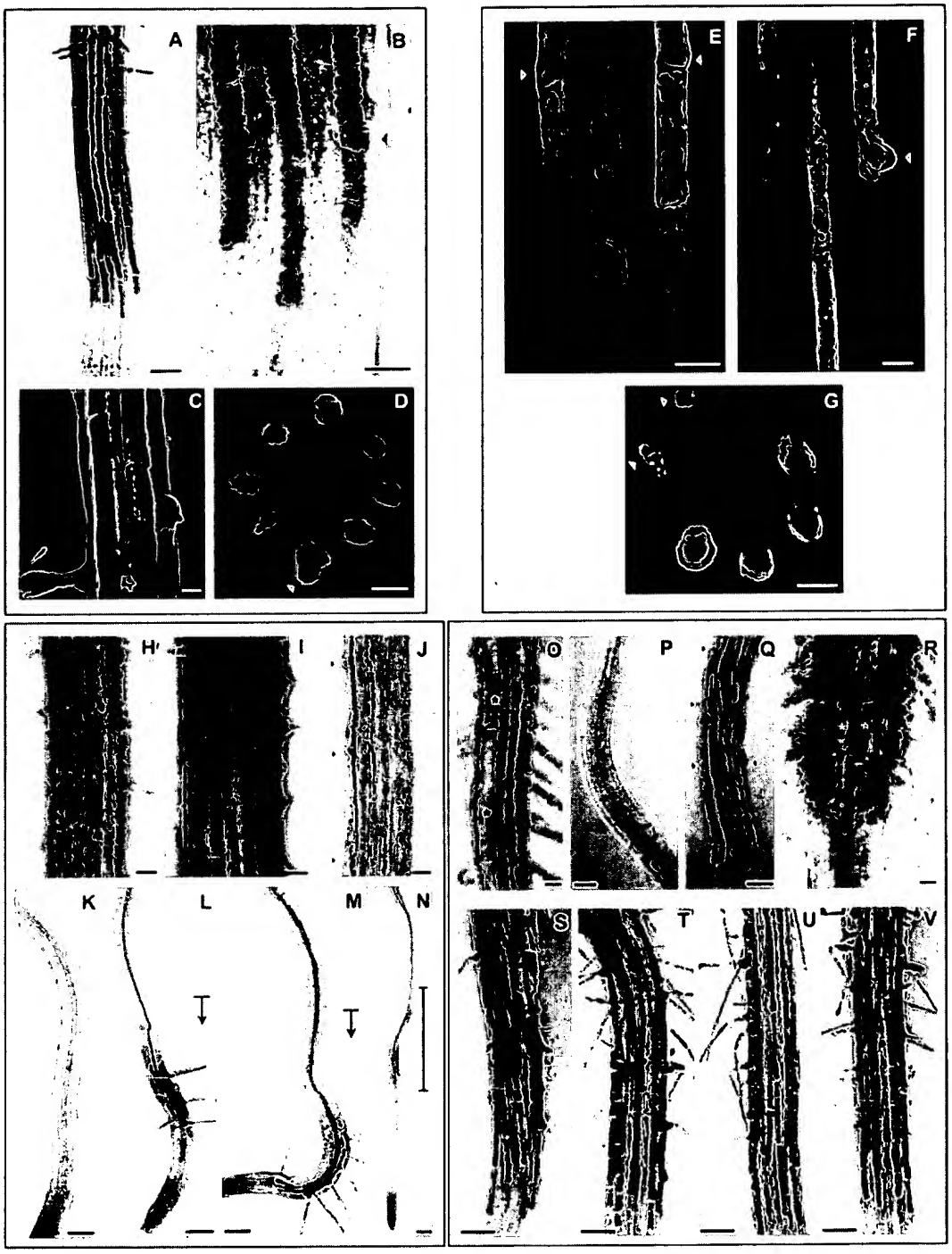

Figure 2. Root Hair Cell–Specific Expression Pattern of *AtEXP7* in the Arabidopsis Root.

(A), **(B)**, and **(H)** to **(V)** show *AtEXP7* promoter::*GUS* expression; **(C)** and **(D)** show *AtEXP7* promoter::*GFP* expression; and **(E)** to **(G)** show *AtEXP7* promoter::genomic *AtEXP7-GFP* expression.

(A) to **(D)** In the wild-type root, reporter gene expression occurs in the root hair cell files. The weaker blue staining between the strong stains are from the hair cell files of the opposite side. **(C)** shows an optical longitudinal section demonstrating GFP expression at the root hair cell files. The

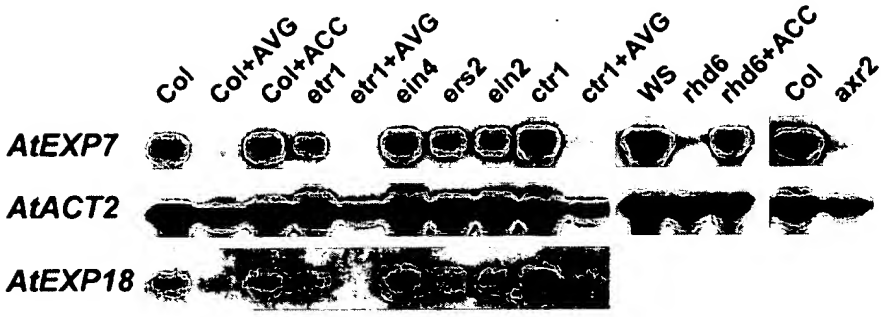

Figure 3. RNA Gel Blot Analyses of *AtEXP7* and *AtEXP18* Transcripts in Different Mutant Backgrounds and under Treatment with Ethylene Precursor and Inhibitor.

Total RNA was prepared from roots of 4-day-old wild-type and mutant seedlings. For ACC (5 μM) and AVG (5 μM) treatments, the seedlings were transferred to chemical-containing plates on day 3. Ten micrograms of total RNA, except for Wassilewskija and *rhd6* (30 μg), was analyzed. The transcript level of Arabidopsis actin2 (*AtACT2*) served as a loading control. Col, Columbia wild type; WS, Wassilewskija wild type.

receptors. We chose 1-MCP as an antagonist of ethylene action because of its high specificity of action and lack of deleterious side effects (Sisler et al., 1996; Hall et al., 2000). At 1 μL/L, 1-MCP almost completely abolished ACC-induced root hair formation and the expression of *AtEXP7* and *AtEXP18* in *rhd6* (Figure 4).

1-MCP Inhibits Auxin- or Root Separation–Induced Root Hair Formation and Expression of Root Hair Expansin Genes

To investigate the possible involvement of ethylene receptors in root hair formation and expansin gene expression induced by auxin or root separation from the agar medium, the antagonism of these effectors by 1-MCP was investigated in the *rhd6* background. Auxin- or root separation–induced root hair formation was greatly inhibited by 1-MCP (1 μL/L). No root hair bulges or elongated root hairs were observed in mutant seedlings treated with indole 3-acetic acid (IAA; 30 nM) together with 1-MCP (Figure 5B). Similarly, 1-MCP inhibited 90% of the root hair formation induced by root separation (Figures 6B and 6C). Consistent with these results, 1-MCP inhibited 70 to 90% of IAA- or root separation–induced expression of *AtEXP7* and *AtEXP18* (Figures 5C to 5H and 6D to 6I). These results show that the coordinate induction of root hairs and expansin gene expression by auxin and root separation requires ethylene sensing, most likely because ethylene is part of the signaling pathway for these effects.

Figure 2. (continued).

red area from propidium iodide indicates the cell boundary. **(D)** shows an optical cross-section of the root demonstrating gene expression at the eight root hair cells. The arrowheads in **(B)** and **(D)** indicate emerging root hair bulges.

(E) to **(G)** Expression of the AtEXP7-GFP fusion protein shows the same pattern as expression of GUS or GFP alone. **(G)** shows an optical cross-section. Arrowheads indicate emerging root hair bulges.

(H) and **(I)** In the *ttg-1* **(H)** and *gl2-1* **(I)** backgrounds, reporter gene expression is observed in cells from both the H and N positions.

(J) *axr2-1* background. Arrowheads indicate some root hair bulges.

(K) to **(N)** *rhd6* background with no treatment **(K)** or with 5 μM ACC **(L)**, 30 nM IAA **(M)**, or separation of the root from the medium **(N)**. The bases of the arrows in **(L)** and **(M)** indicate the approximate starting points of hormone treatments. The vertical bar in **(N)** indicates where the root was separated from agar.

(O) to **(Q)** Wild-type roots treated with 5 μM ACC **(O)**, 5 μM AVG **(P)**, or 50 μM silver ion **(Q)**. Stars in **(O)** indicate ectopic expression of GUS in the N positions.

(R) *ctr1-1* background. Stars indicate ectopic expression of GUS in the N positions.

(S) to **(U)** Dominant ethylene receptor mutants *etr1-1* **(S)**, *ein4* **(T)**, and *ers2-1* **(U)**.

(V) *ein2-1* background.

Bars = 100 μm in **(K)** to **(N)**, 50 μm in **(A)**, **(P)**, **(Q)**, and **(S)** to **(V)**, and 20 μm in **(B)** to **(J)**, **(O)**, and **(R)**.

Table 1. Root Hair Number in Wild-Type and Mutant Plants with Ethylene Precursor or Inhibitor Treatment

	Percent of Total Root Hair Cells[a]				Percent of Root Hair Cells in the N position[b]	
Plant	No Treatment	ACC (5 μM)	AVG (5 μM)	1-MCP (1 μL/L)	No Treatment	ACC (5 μM)
Columbia	51.1 ± 3.3	65.9 ± 5.8	1.3 ± 2.3	44.0 ± 4.2	1.1 ± 3.3	15.9 ± 5.8
etr1-1	45.8 ± 6.7	48.1 ± 3.7	0 ± 0	43.0 ± 2.7	2.7 ± 3.3	2.5 ± 4.6
etr2	51.3 ± 2.3	51.5 ± 3.4	0.6 ± 1.8	48.3 ± 2.5	1.3 ± 2.3	1.5 ± 3.4
ers1	55.4 ± 6.2	52.5 ± 4.2	0.6 ± 1.8	49.5 ± 2.7	5.4 ± 6.2	2.5 ± 4.2
ers2-1	50.0 ± 3.0	55.0 ± 8.2	0 ± 0	44.1 ± 3.8	0.8 ± 1.9	6.4 ± 8.5
ein4	50.4 ± 1.4	57.2 ± 8.7	0 ± 0	42.3 ± 3.5	0.4 ± 1.4	7.2 ± 8.7
ein2-1	48.8 ± 3.8	45.8 ± 2.0	0 ± 0	N.D.[c]	2.5 ± 3.4	0 ± 0
ctr1-1	65.0 ± 6.4	N.D.	0 ± 0	N.D.	15.0 ± 6.4	N.D.
eto2	63.8 ± 4.8	N.D.	0 ± 0	N.D.	13.8 ± 4.8	N.D.
etr1-7	50.7 ± 5.1	N.D.	N.D.	N.D.	0.7 ± 5.1	N.D.
rhd6	0 ± 0	37.6 ± 13.9	N.D.	N.D.	0 ± 0	3.7 ± 6.6

Values shown are means ± SD (n = 140 to 260).

[a] Percentage of root hair–bearing epidermal cells among total epidermal cells counted, including cells in both the H and N positions.

[b] Percentage of root hair–bearing epidermal cells at the N position among total epidermal cells counted.

[c] N.D., not determined.

Endogenous Ethylene Is Not Involved in Normal (Default) Root Hair Formation and Expression of Root Hair Expansin Genes in the Wild Type

To verify the role of endogenous ethylene during root hair formation and expression of root hair expansin genes, we examined the effects of dominant mutations of ethylene receptors and inhibitors of ethylene action. Here, we use the term "endogenous ethylene" to designate the internal ethylene level in the plant without any mutations or treatments that would induce the overproduction of ethylene.

Our results showed that mutations in the ethylene signaling pathway failed to inhibit root hair formation and expansin gene expression. None of the five dominant-negative ethylene receptor mutants showed a significant reduction in root hair density (Table 1). The *ein2* mutant, which is known to exhibit the strongest ethylene phenotype, also had a normal number of root hairs, consistent with a previous report (Masucci and Schiefelbein, 1996). *AtEXP7* expression also was patterned normally in roots of the ethylene mutants (Figures 2S to 2V), and expression levels were not reduced greatly in the mutant backgrounds (Figure 3).

Aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, has been used to test the role of ethylene in root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995). Our results showed that 5 μM AVG almost completely blocked root hair formation and *AtEXP7* expression in the wild type (Figures 2P and 3, Table 1). However, surprisingly, AVG (5 μM) almost completely inhibited root hair formation in the constitutively ethylene-responsive mutant *ctr1-1* (Table 1), even though this mutant should not respond to AVG inhibition of ethylene synthesis. AVG markedly repressed the expression of *AtEXP7* in *ctr1-1* and other genotypes, but it also reduced actin gene expression (*AtACT2*; Figure 3). Although it was reported that ACC could partially restore root hair formation in the AVG-treated root (Masucci and Schiefelbein, 1994, 1996), our results indicate that AVG has significant deleterious effects on root hair development. Toxicity of AVG also is reported in root formation (Jackson, 1991) and somatic embryogenesis (Meijer, 1989). This may occur because AVG, functioning as an inhibitor of pyridoxal phosphate–dependent enzymes (Abel, 1985), probably interferes with other biochemical processes that are vulnerable to the inhibitor, not only ethylene biosynthesis.

To further test the role of endogenous ethylene during root hair formation in wild-type plants, we used 1-MCP, which binds to multiple ethylene receptors (Hall et al., 2000). Thus, we expected that 1-MCP would strongly inhibit root hair formation in wild-type plants if endogenous ethylene were involved. However, root hair formation in the wild-type root was inhibited very little by 1 μL/L 1-MCP (Table 1) or even by 10 μL/L (data not shown). By contrast, 0.22 μL/L 1-MCP showed saturated inhibitory effects on both ethylene binding to the receptors and the triple response (Hall et al., 2000). 1-MCP also did not significantly inhibit the expression of *AtEXP7* and *AtEXP18* in the wild type (Figure 7). 1-MCP is not able to reverse the constitutive ethylene-responsive phenotype of *ctr1* (Hall et al., 2000), in contrast to the deleterious effect of AVG on the *ctr1* root.

Silver ion (an inhibitor of ethylene perception) at 50 μM did not abolish root hair formation and *AtEXP7* expression, although it completely inhibited hair elongation (Figure 2Q). A previous study reported that silver ion (1 μM) greatly reduced root hair number (Tanimoto et al., 1995),

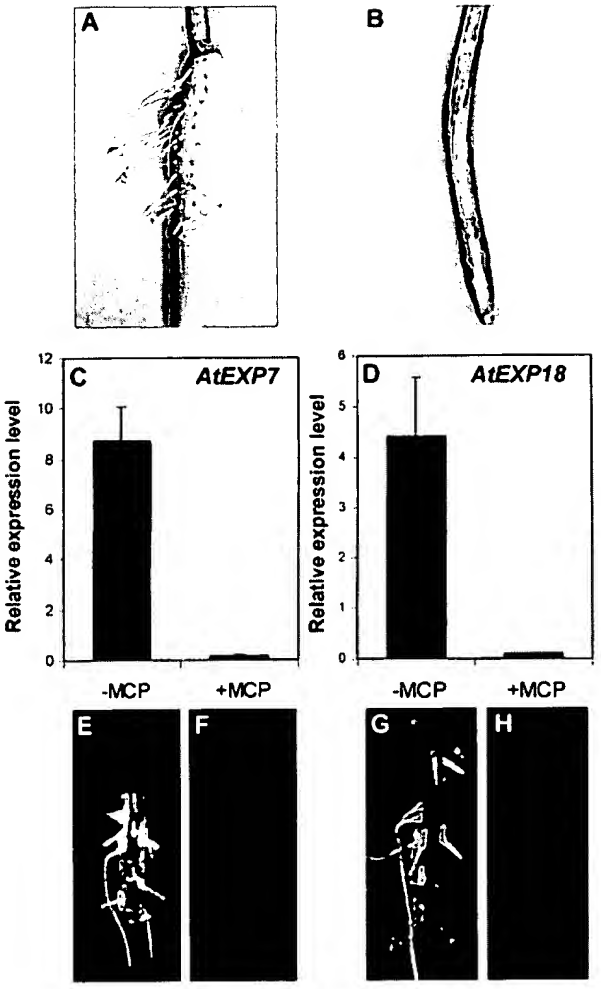

Figure 4. Effect of 1-MCP on ACC-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and **(B)** Bright-field microscopy images of roots grown in 5 μM ACC without **(A)** or with **(B)** 1 μL/L 1-MCP.

(C) and **(D)** Relative expression levels of *AtEXP7* **(C)** and *AtEXP18* **(D)** in the root when induced by 5 μM ACC without (−MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors (n = 11 to 18).

(E) to **(H)** Confocal microscopy images of the roots harboring *AtEXP7* promoter::*GFP* **([E]** and **[F])** and *AtEXP18* promoter::*GFP* **([G]** and **[H])**. Seedlings were incubated in 5 μM ACC without **([E]** and **[G])** or with **([F])** and **[H])** 1-MCP.

but it is not clear whether small bulges were counted. The effects of ACC, AVG, and mutations in ethylene signaling on the expression of *AtEXP18* also resembled those on *AtEXP7* expression, as shown by RNA gel blot analysis (Figure 3).

Endogenous Ethylene Affects Root Hair Elongation

In contrast to root hair initiation, ethylene showed an unambiguous effect on root hair elongation, consistent with a

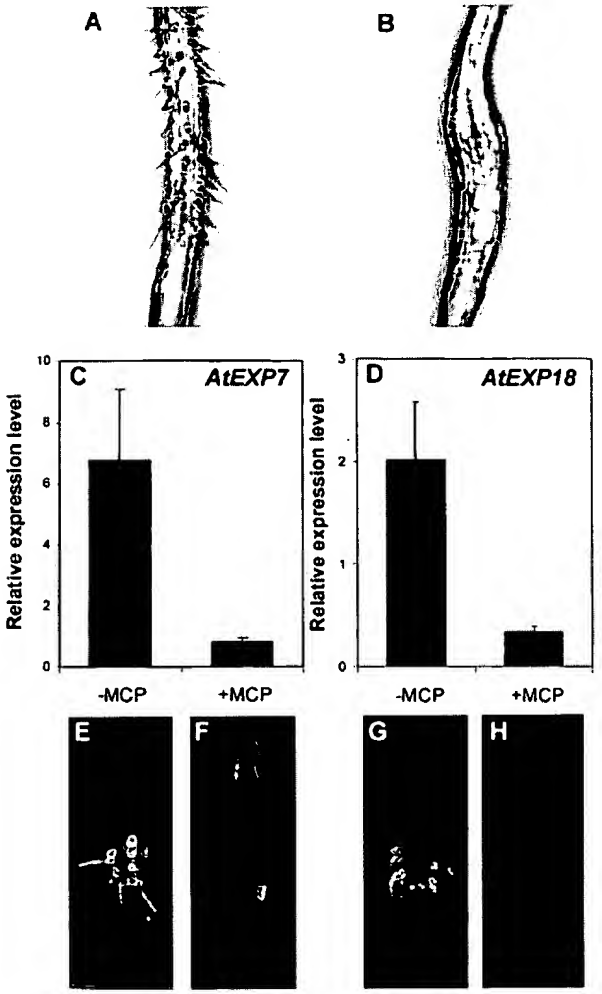

Figure 5. Effect of 1-MCP on IAA-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and **(B)** Bright-field microscopy images of the roots grown in 30 nM IAA without **(A)** or with **(B)** 1 μL/L 1-MCP.

(C) and **(D)** Relative expression levels of *AtEXP7* **(C)** and *AtEXP18* **(D)** in the root when induced by IAA without (−MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors (n = 7 to 12).

(E) to **(H)** Confocal microscopy images of the roots harboring *AtEXP7* promoter::*GFP* **([E]** and **[F])** and *AtEXP18* promoter::*GFP* **([G]** and **[H])**. Seedlings were incubated in IAA without **([E]** and **[G])** or with **([F]** and **[H])** 1-MCP.

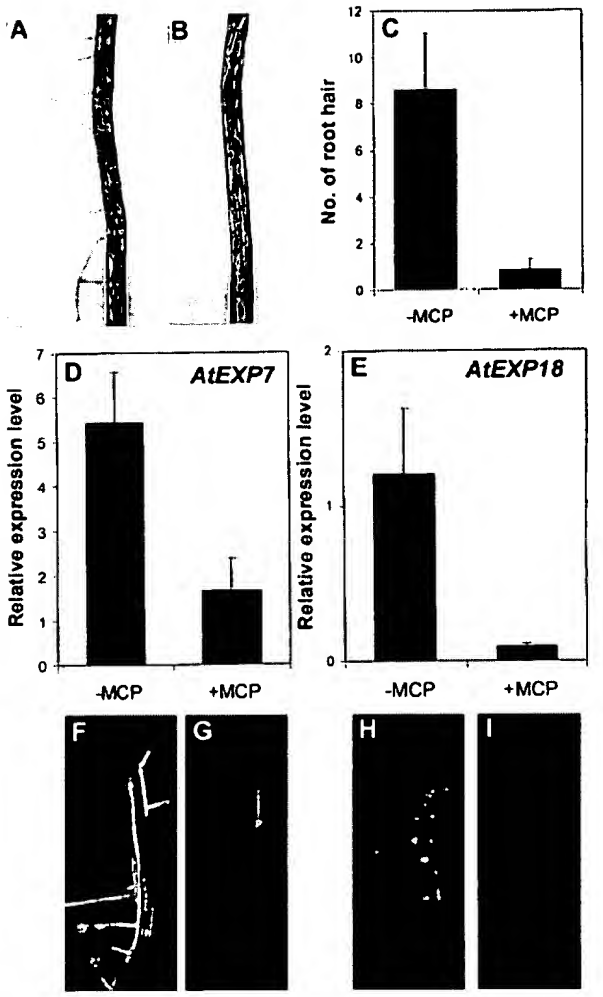

Figure 6. Effect of 1-MCP on Root Separation-Induced Root Hair Formation and Expansin Gene Expression in the *rhd6* Root.

(A) and **(B)** Bright-field microscopy images of the roots separated from the agar medium without **(A)** or with **(B)** 1 μL/L 1-MCP.

(C) Effect of 1-MCP on root hair number in separation-treated roots. Total root hairs were counted from the separated region of the root. Bars indicate standard errors (n = 13 to 19).

(D) and **(E)** Relative expression levels of *AtEXP7* **(D)** and *AtEXP18* **(E)** in the root when induced by separation of the root without (−MCP) or with (+MCP) 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter or the *AtEXP18* promoter. Bars indicate standard errors (n = 11 to 15).

(F) to **(I)** Confocal microscopy images of roots harboring *AtEXP7* promoter::*GFP* **([F]** and **[G])** and *AtEXP18* promoter::*GFP* **([H]** and **[I])**. Seedlings whose roots were separated from the medium were incubated without **([F]** and **[H])** or with **([G]** and **[I])** 1-MCP.

previous report (Pitts et al., 1998). Root hair length was decreased significantly in four dominant ethylene receptor mutants (Table 2). Treatment with 1-MCP also greatly decreased root hair elongation in the wild type. Considering the effect of each dominant mutation on root hair length, we can assess the cell type–specific roles of the five ethylene receptors. ETR1 seems to play the most significant role in root hair elongation, followed by ERS1 ≥ ERS2 > ETR2. EIN4 appears to have no function in root hair elongation.

Promoter Analyses of *AtEXP7* and *AtEXP18*

AtEXP7 and *AtEXP18* are expressed specifically in the root hair cell and are induced by ethylene, auxin, and separation of the root from the medium. To define the regulatory elements for the hair cell specificity and effector inducibility of the promoter, we performed promoter analyses of the genes by sequential deletion of the 5′ regions, nucleotide substitution, and gain of function of the *cis* elements. The deleted or substituted promoters were fused directly to the GFP coding sequence, and the gain-of-function *cis* elements were combined with the 35S minimal promoter region of *Cauliflower mosaic virus* (−64 35S promoter; Eyal et al., 1995) that was followed by the GFP sequence. For unambiguous evaluation of the promoter activities, the promoter::*GFP* constructs were introduced stably into plants (wild type and *rhd6*) by Agrobacterium transformation. To assess the inducibility of promoter activities by ethylene, auxin, and root separation, we treated the transformed *rhd6* plants with 5 μM ACC, 30 nM IAA, or separation of the root. Promoter activity was evaluated by confocal laser scanning microscopy to measure GFP fluorescence in roots of the first generation of transformants (9 to 62 independent T1 lines per construct, with an average of 28). The histogram function of Adobe Photoshop was used to quantify the relative GFP fluorescence.

For 5′ deletion analysis of the *AtEXP7* promoter, sequential deletions from −1380 to +48 bp, relative to the predicted transcription initiation site, were generated (Figure 8A). Deletions to −386 bp did not significantly affect the promoter activity in either the wild type or *rhd6* treated with ACC (Figures 8B and 8C). Further deletion to −245 bp decreased promoter activity by 50 to 70% in both backgrounds, and this level continued through additional deletions to −134 bp. In auxin-treated *rhd6* transformants, the promoter activity decreased gradually in deletions from −386 to −134 bp, where ~50% of the activity remained (Figure 8D). Root separation treatment of *rhd6* also gave a similar result, except that the deletion to −386 bp decreased the promoter activity significantly (Figure 8E).

Although the promoter activity was reduced considerably by deletion to −245 bp, both the cell specificity and the inducibility by effectors were maintained until deletion to −134 bp, and no novel expression patterns were observed in other tissues (data not shown). The cell specificity and the

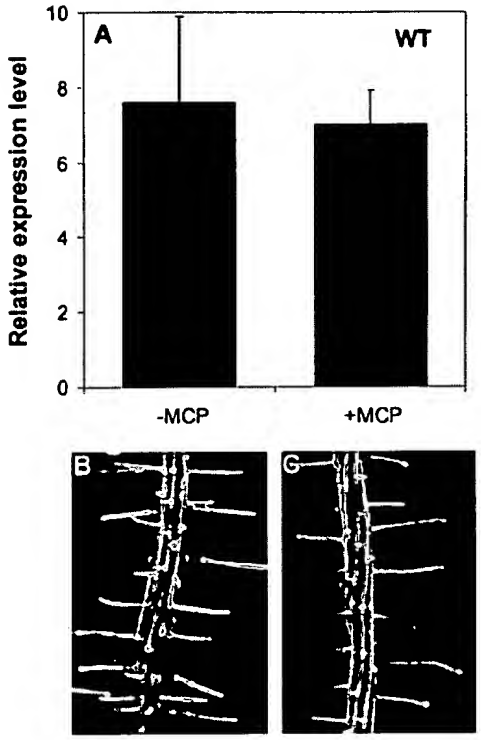

Figure 7. Effect of 1-MCP on Expansin Gene Expression and Root Hair Formation in the Wild Type.

(A) Relative expression levels of *AtEXP7* without (−MCP) or with (+MCP) 1 μL/L 1-MCP. Relative expression levels were evaluated from GFP expression (fluorescence) driven by the *AtEXP7* promoter. Bars indicate standard errors (n = 7 to 10). WT, wild type.
(B) and **(C)** Confocal microscopy images of roots harboring *AtEXP7* promoter::*GFP* without **(B)** or with **(C)** 1 μL/L 1-MCP.

inducibility by effectors disappeared completely with deletion to −70 bp. Although elements for auxin (TGTCTC; −808 bp) and ethylene (AATTCAAA; −615 bp) response are located on the *AtEXP7* promoter, deletions of those elements did not affect the responsiveness of the promoter to these hormones (Figures 8C and 8D). Deletion analysis of the *AtEXP7* promoter suggested that the elements for cell specificity and inducibility by these effectors are located between −134 and −70 bp. In this region (Figure 9A) are three repeats of a core binding sequence (AAAG) for the DOF zinc finger protein (Yanagisawa and Schmidt, 1999) and one core motif (GGATA) for MYBSt1, a MYB-like protein (Baranowskij et al., 1994). The distal region between −386 and −245 bp likely contains some enhancing elements, because deletion of this region reduced promoter activity significantly. A MYBSt1 core motif also is found in this distal promoter region (−281 to −276 bp).

To define the *cis*-regulatory elements in the proximal region (−134 to −70 bp) of the *AtEXP7* promoter, seven 9- to 10-bp-long substitution mutations were introduced into this region. To acquire the greatest mutational effects, an A/T base pair was changed to G/C or C/G. Substitution mutations by ~9 to 10 bp are small enough to localize the controlling elements with reasonable precision (Carey and Smale, 2000). These substitutions replaced the DOF and MYBSt1 core elements and their flanking regions. The substitution mutations E7M1~E7M7 were produced from the −386-bp deletion so that the wild-type promoter had full activity (Figure 9A). Although promoter activity fluctuated between 50 and 130% compared with wild-type (−386 bp) activity, the substitutions E7M1~E7M5 did not greatly diminish promoter activity in either wild-type or ACC-treated *rhd6* roots (Figures 9B and 9C). However, both E7M6 (which includes the MYBSt1 core element) and E7M7 (flanking E7M6) decreased the activity to ~13 to 26%. Similar results were obtained by treatment with auxin or root separation (data not shown). These results suggest that the 19-bp motif containing the MYBSt1 element (hereafter called the −80/−62 element) is most important for both hair cell specificity and inducibility by ethylene, auxin, and root separation.

A gain-of-function analysis was performed to confirm that the identified elements are able, in isolation, to direct hair cell specificity. E7G1~E7G3 are short sequences that contain the proximal MYBSt1 core with different 3′ extensions, and E7G4 includes the entire proximal region between −134 and −46 bp. E7G4M6 and E7G4M7 are the same as E7G4 except that they harbor E7M6 and E7M7 substitution mutations, respectively (Figure 9A). The results shown in Figures 9D to 9G are from the wild-type background, but similar results were obtained with ACC-treated *rhd6* (data not shown). The 35S minimal promoter (mp35S) alone did not show GFP expression (Figure 9E). The gain-of-function promoter constructs E7G1~E7G3 showed promoter activity

Table 2. Root Hair Length in Wild-Type and Ethylene Mutant Plants with Ethylene Precursor or Inhibitor Treatment

Plant	No Treatment	ACC (5 μM)	1-MCP (1 μL/L)
Columbia	0.91 ± 0.22	1.07 ± 0.14	0.51 ± 0.19
etr1-1	0.26 ± 0.14	0.21 ± 0.20	0.27 ± 0.16
etr2	0.72 ± 0.20	0.66 ± 0.08	0.65 ± 0.09
ers1	0.40 ± 0.14	0.41 ± 0.12	0.39 ± 0.13
ers2-1	0.47 ± 0.20	0.47 ± 0.19	0.35 ± 0.11
ein4	0.92 ± 0.12	0.95 ± 0.12	0.56 ± 0.13
ein2-1	0.04 ± 0.02	0.05 ± 0.03	N.D.[a]
etr1-7	0.82 ± 0.07	N.D.	N.D.
ctr1-1	1.22 ± 0.18	N.D.	N.D.

Values shown are means ± SD in mm (n = 35).
[a] N.D., not determined.

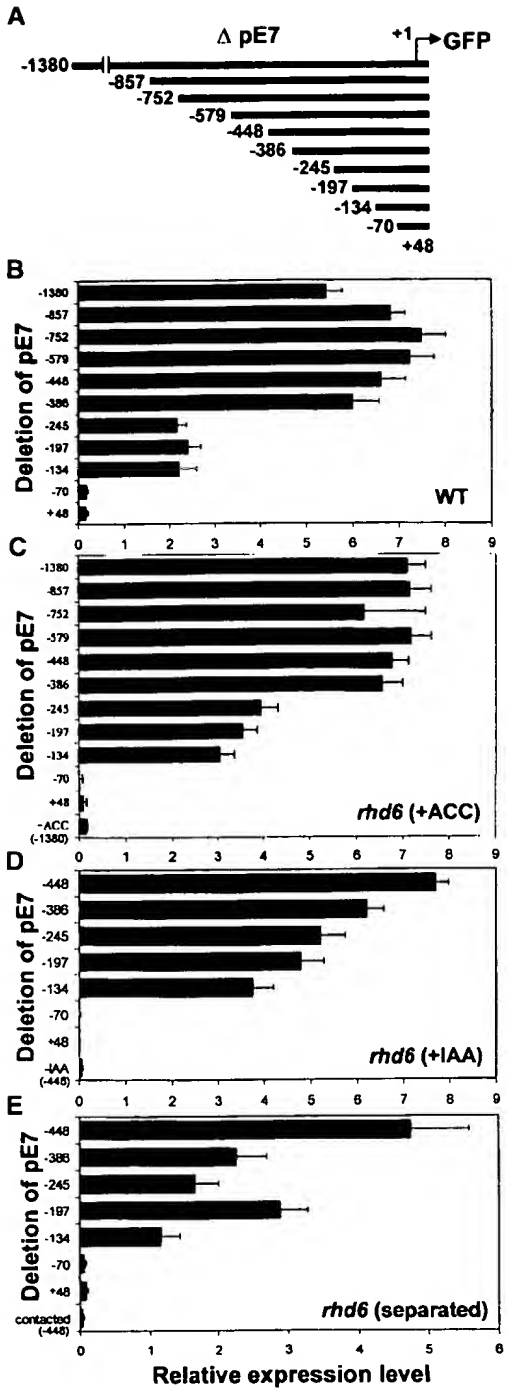

Figure 8. Deletion Analysis of the *AtEXP7* Promoter.

(A) Deletions of the *AtEXP7* promoter (ΔpE7) that are fused to the coding region of GFP. Numbers indicate nucleotide positions relative to the transcription initiation site (+1).

(B) Relative activities (GFP expression) of the truncated *AtEXP7* promoters in the wild-type (WT) root. Bars indicate standard errors.

as weak as that of mp35S (Figure 9D), but 20 to 30% of T1 lines from these constructs showed very low and irregular GFP fluorescence in root hair cells (see supplemental data online), which was undetectable in mp35S roots. No significant differences in promoter strength among E7G1~E7G3 were found. By contrast, E7G4 could direct strong hair cell–specific expression of the reporter gene (Figures 9D and 9F). However, the substitution mutation of the E7G4 promoter fragment at the E7M6 or E7M7 site eliminated the promoter activity almost completely (Figures 9D and 9G). This gain-of-function promoter analysis demonstrates that the −80/−62 element confers the hair cell specificity of the *AtEXP7* promoter. However, some additional elements in the proximal region, particularly between −134 and −81 bp, seem to be required for strong promoter activity. These additional elements could be functionally redundant, because the individual substitution mutations (E7M1~E7M5) elsewhere than in the −80/−62 region did not reduce promoter activity substantially (Figures 9B and 9C).

For analysis of the *AtEXP18* promoter, deletions between −1016 and +42 bp were generated (Figure 10A). The *AtEXP18* promoter activity maintained its full strength until the deletion to −241 bp and showed an ~50% decrease by further deletion to −196 bp in both wild-type and ACC- or IAA-treated *rhd6* seedlings (Figures 10B to 10D). The deletions beyond −145 bp completely eliminated the promoter activity in both the wild type and *rhd6* with effector treatments. A similar change of promoter activity was observed in root separation–treated *rhd6*, except that the deletion to −321 bp reduced the activity significantly (Figure 10E). Deletion analysis of the *AtEXP18* promoter indicated that the −196/−145 region contains elements for hair cell specificity and the −241/−196 region may include some enhancing elements relevant to promoter strength. The −196/−145 region of *AtEXP18*, resembling the −80/−62 element of *AtEXP7*, is likely the target of signals from ethylene, auxin, and root separation, because these effectors all require this region for gene induction (Figure 11). However, the −196/−145 region of *AtEXP18* does not contain the MYBSt1 binding motif or similar sequences found in the −80/−62 element of *AtEXP7*. This difference indicates that the cell specificity of the two promoters probably is determined by different transcription factors, which nevertheless are regulated similarly by developmental factors, auxin, ethylene, and root separation.

(C) to **(E)** Relative activities of the truncated *AtEXP7* promoters in the *rhd6* root. For gene induction, the transformed mutant seedlings were treated with 5 μM ACC **(C)** or 30 nM IAA **(D)** or roots were separated from agar to expose them to air **(E)** for 1 day before observation. Bars indicate standard errors.

In **(B)** to **(E)**, n = 27 to 62.

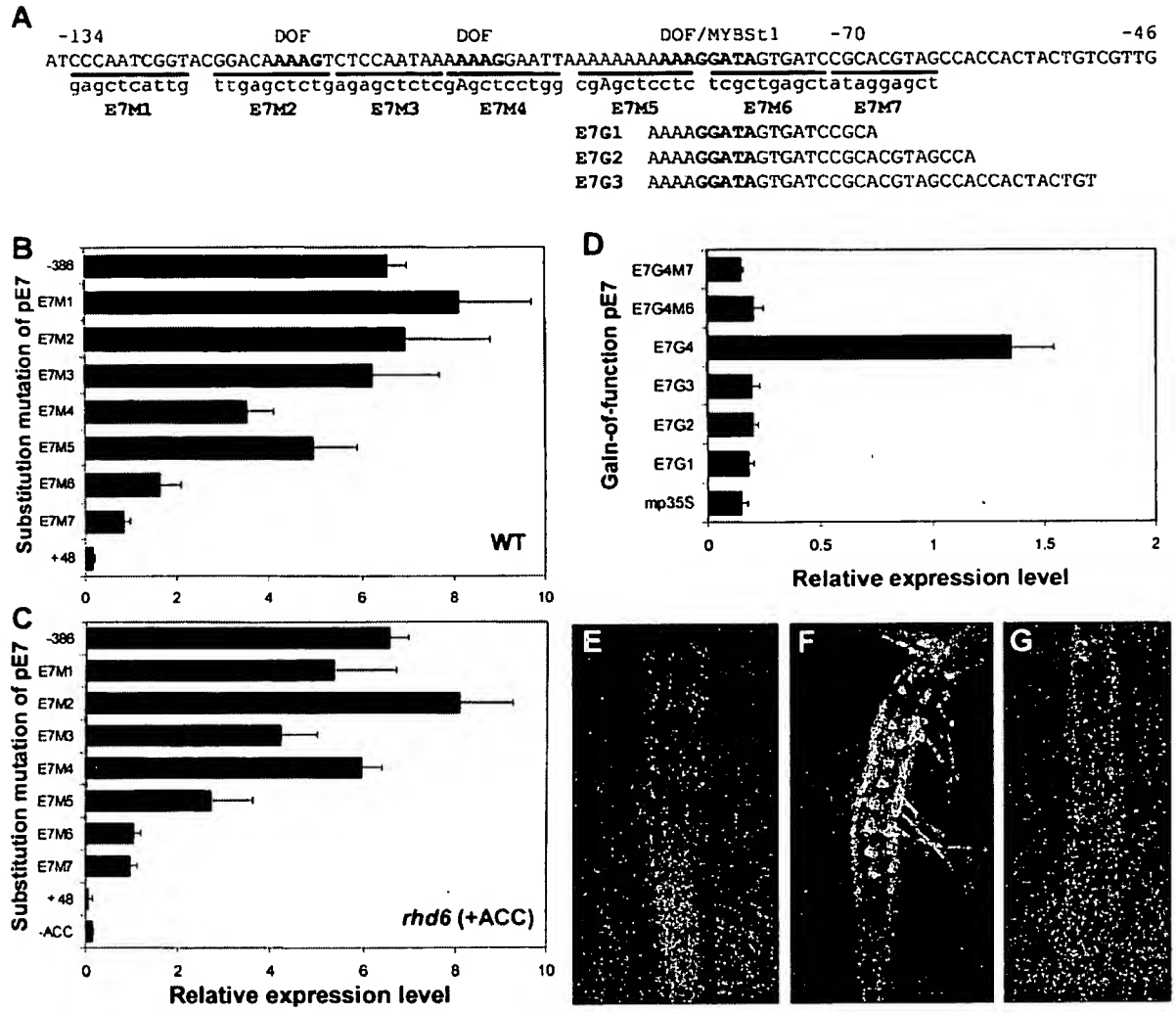

Figure 9. Substitution and Gain-of-Function Analyses of the *AtEXP7* Promoter.

(A) The proximal promoter region of *AtEXP7* between −134 and −46 bp. For substitution mutations (E7M1∼E7M7), the underlined regions were replaced by the nucleotides shown in lowercase letters. These substitution mutations were generated from the region between −386 and +48 bp. E7G1∼E7G3 are the gain-of-function promoter fragments. The substituted promoters were fused to the coding region of GFP, and the gain-of-function promoter fragments were connected to the minimal 35S promoter of *Cauliflower mosaic virus* (mp35S) before the GFP gene. The putative DOF (AAAG) and MYBSt1 (GGATA) core motifs are indicated.

(B) and **(C)** Relative activities (GFP expression) of the substituted *AtEXP7* promoters in the wild-type (WT) root **(B)** and in the *rhd6* root with 5 μM ACC treatment **(C)**. Bars indicate standard errors (n = 15 to 32).

(D) Relative activities of the gain-of-function *AtEXP7* promoters in the wild-type root. Bars indicate standard errors (n = 9 to 14). E7G4 contains the −134/−46 region (wild-type promoter), and E7G4M6 and E7G4M7 are the same as the E7G4 construct but with E7M6 and E7M7 substitution mutations, respectively.

(E) to **(G)** Confocal microscopy images of roots harboring the gain-of-function *AtEXP7* promoters mp35S **(E)**, E7G4 **(F)**, and E7G4M6 **(G)** (a similar expression pattern was observed with E7G4M7).

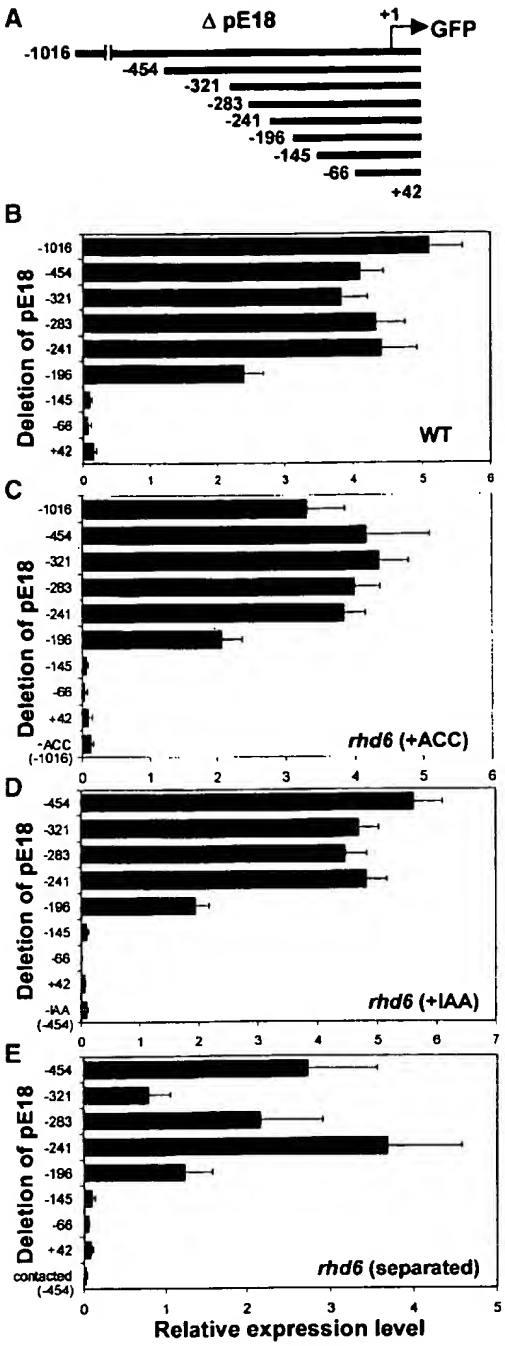

Figure 10. Deletion Analysis of the *AtEXP18* Promoter.

(A) Deletions of the *AtEXP18* promoter (ΔpE18) that are fused to the coding region of GFP. Numbers indicate nucleotide positions relative to the transcription initiation site (+1).

(B) Relative activities (GFP expression) of the truncated *AtEXP18* promoters in the wild-type (WT) root. Bars indicate standard errors.

(C) to **(E)** Relative activities of the truncated *AtEXP18* promoters in the *rhd6* root. For gene induction, the transformed mutant seedlings were treated with 5 μM ACC **(C)** or 30 nM IAA **(D)** or roots were separated from the agar medium **(E)** for 1 day before observation. Bars indicate standard errors.

In **(B)** to **(E)**, n = 25 to 40.

DISCUSSION

Ethylene and Root Hair Development

Recent studies have contributed significantly to our understanding of cell fate determination in the Arabidopsis root epidermis. However, the morphogenetic process of root hair development, which is regulated by hormones and environmental factors, has remained less characterized. In this study, we examined the role of endogenous ethylene and the hierarchical relationship between ethylene, auxin, and an environmental factor (root separation from the agar medium) in root hair initiation. To understand the action of these factors at the gene regulation level, we adopted two expansin genes, *AtEXP7* and *AtEXP18*, whose expression is linked tightly to root hair initiation, as molecular markers.

The involvement of ethylene in root hair formation has been demonstrated in genetic and pharmacological studies. Treatment with the ethylene precursor ACC and mutations of *ctr1* and *eto* induced additional root hairs from the cells in the N position (Dolan et al., 1994; Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995; Cao et al., 1999) (Table 1), and these factors also could restore root hairs in the root hair–defective *rhd6* mutant (Masucci and Schiefelbein, 1996). Although these results clearly show that ethylene is a positive effector of root hair formation, they are indicative of the effect of a constitutive ethylene response and excessive (or exogenous) ethylene but not of the effect of the normal endogenous ethylene level. The mutation of *CTR1* causes constitutive ethylene responses regardless of the absence or presence of ethylene, and the *eto* mutants produce excessive ethylene from 2- to 100-fold (Kieber et al., 1993).

The ethylene biosynthesis inhibitor AVG has been used to show the role of endogenous ethylene, which greatly inhibits root hair formation (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995) (Table 1). However, AVG likely has toxicity to root hair development, because it completely inhibited root hair formation and the expression of *AtEXP7* and *AtEXP18*, even in the *ctr1* mutant (Figure 3, Table 1).

The role of endogenous ethylene in the wild type can be assessed by the use of mutations that block the ethylene responses. A previous study reported that the dominant ethylene receptor mutant *etr1* maintains normal root hair density (Masucci and Schiefelbein, 1996), thereby raising doubt about the role of endogenous ethylene during the normal (default) process of root hair formation. In Arabidopsis, there are five ethylene receptors whose physiological function, in

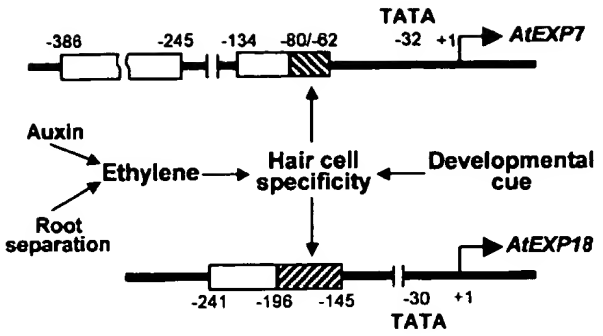

Figure 11. Summary of Promoter Analyses of *AtEXP7* and *AtEXP18*.

The hatched boxes represent elements for hair cell specificity, and the open boxes represent elements that are likely to be relevant to promoter strength. The environmental (root separation) and hormonal signals converge on the elements for hair cell specificity. Numbers indicate nucleotide positions relative to the transcription initiation site (+1). TATA indicates the TATA box.

terms of the triple response, is similar. Dominant mutations in these receptors negatively regulate ethylene responses by constitutively activating CTR1, the negative regulator of downstream ethylene responses. Thus, a dominant mutation in any one of the receptors is able to suppress ethylene responses (Hua and Meyerowitz, 1998). In spite of this genetic principle, inhibition by the dominant mutation shows a dosage-dependent response according to the number of mutant loci and also shows different degrees of phenotypic effect among the five receptors (Hall et al., 1999). Therefore, the contribution of ethylene receptors to root hair formation might depend on receptor species and their temporal/spatial expression pattern.

To determine whether ethylene receptors other than ETR1 are involved in root hair formation, we examined the effect of dominant mutations in all five ethylene receptors. Furthermore, because multiple receptors might be involved in root hair formation, the specific ethylene antagonist 1-MCP was used to simultaneously inhibit ethylene binding by different ethylene receptors. Our results showed that neither the dominant mutations of ethylene receptors nor 1-MCP treatment substantially reduced root hair numbers and the expression of *AtEXP7* and *AtEXP18* (Figures 3 and 7, Table 1), indicating that endogenous ethylene is not required for normal (default) root hair formation in the wild type.

Ethylene, however, is likely to mediate auxin- or root separation–induced root hair formation. Blocking the ethylene perception by 1-MCP almost completely inhibited auxin- or root separation–induced root hair formation and expression of *AtEXP7* and *AtEXP18* (Figures 5 and 6). Auxin and certain biotic/abiotic factors, such as pathogens, wounding, chilling, hypoxia, and water stress, are well-known stimulators of ethylene biosynthesis (McKeon et al., 1995). Localized water stress could develop in the root when it is separated from the agar medium or exposed to air, a treatment that is known to stimulate root hair elongation (Okada and Shimura, 1994). Therefore, auxin and root separation may induce root hair initiation through an increase in ethylene production, although we do not exclude the possibility that these treatments affect components of ethylene signaling.

A previous study suggested that ethylene and auxin take separate pathways to affect root hair development. Auxin restored root hairs in the AVG-treated root and in the *aux1 etr1* double mutant (Masucci and Schiefelbein, 1996). However, the latter case indicates a complicated aspect of root hair development, because ACC significantly suppressed root hair formation in the double mutant rather than simply having no effect on the restoration of root hairs. A similar perplexing result from the same study is that ACC also inhibited root hair formation considerably in the *rhd6 ein2* double mutant. It appears that excessive ethylene (or its precursor) inhibits the ethylene responses of ethylene-insensitive mutants in certain conditions.

The dominant mutant *axr2* maintains 64% of root hairs compared with the wild type, and ACC or auxin only partially restores the root hair number in the mutant (74 to 81% compared with the wild type) (Masucci and Schiefelbein, 1996). The *axr2* mutant carries the gain-of-function mutation in an Aux/IAA transcriptional repressor (IAA7) so that the mutant molecule is resistant to the auxin-mediated degradation process (Nagpal et al., 2000; Tiwari et al., 2001). The *axr2* plant can be less sensitive to ACC if AXR2/IAA7 represses expression of the components of ethylene signaling or if the gain-of-function mutant protein finds new targets, such as genes required for the root hair initiation machinery, as a result of its durability time and concentration in the nucleus. The epistatic effect of *axr2* over *ttg* or *gl2* (Masucci and Schiefelbein, 1996) could be acquired if the latter case occurs.

In contrast to root hair initiation, root hair elongation is dependent on endogenous ethylene. Blocking ethylene perception by gain-of-function mutations of the ethylene receptors or by 1-MCP markedly inhibited root hair elongation (Table 2). The difference in ethylene action on the initiation and elongation of root hairs leads us to propose that the two responses have different sensitivities to ethylene. Root hair initiation may require a higher ethylene level than does the root hair elongation process. Treatment with ACC, auxin, or other stimuli is required to exceed the ethylene concentration needed to stimulate root hair initiation, whereas the lower endogenous ethylene level is sufficient to regulate root hair elongation. Alternatively, it is conceivable that ethylene biosynthesis increases during root hair elongation. Genetic studies indicate that different sets of gene products are instrumental for the root hair initiation and root hair elongation steps (Parker et al., 2000; Schiefelbein, 2000). This finding implies that the two ethylene-dependent responses in a single root hair cell result from the activation of different genetic pathways by different ethylene levels.

Two Different Pathways Manipulate Root Hair Initiation

RHD6 is likely to be a major regulator in the developmental pathway (through TTG/GL2) for root hair formation. Defects in the negative regulator TTG or GL2 induce root hairs from the cells in the N position as well as in the H position. However, root hair numbers in *ttg* and *gl2* mutants are reduced greatly by the defect in RHD6, indicating that RHD6 is an important downstream regulator of the TTG/GL2 pathway (Masucci and Schiefelbein, 1996). The fact that auxin, ethylene, and root separation can restore root hairs in *rhd6* led us to a scheme, illustrated in Figure 12, whereby the separate environmental/hormonal signaling pathway converges with the normal developmental pathway downstream of RHD6. We show the environmental signal (root separation from the medium) as separate from the auxin pathway because root separation restores normal root hair growth in the *aux1* mutant (Okada and Shimura, 1994).

However, the environmental/hormonal pathway appears to have a differential influence on the two epidermal positions (H and N). This is seen clearly in the *rhd6* background (Table 1), in which 5 μM ACC stimulated root hair formation in the H position but had negligible effect in the N position. Even higher levels of ACC (50 μM), as well as the *ctr1* and *eto* mutations, induced only some of the cells in the N position to form root hairs (Dolan et al., 1994; Masucci and Schiefelbein, 1996; Cao et al., 1999) (Table 1). This differential response could result from a lower ethylene (or ACC) sensitivity of cells in the N position compared with cells in the H position (Dolan, 1997; Cao et al., 1999).

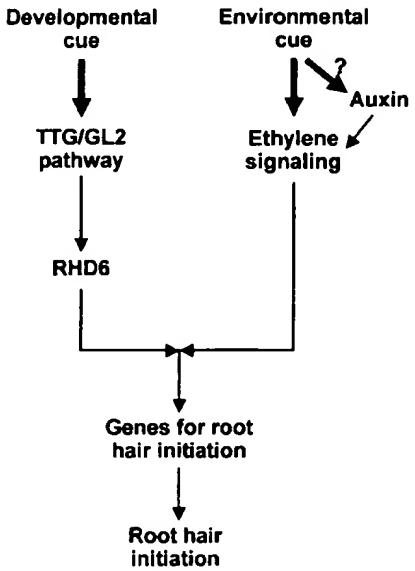

Figure 12. Model Illustrating How Two Separate Signaling Pathways from Developmental and Environmental Cues Merge to Regulate Root Hair Initiation in Arabidopsis.

Arrows designate the information flow.

Because the occasional root hairs in *rhd6* emerge in abnormal cell positions, RHD6 was implicated in the control of hair cell polarity (Masucci and Schiefelbein, 1994). Cell specification seems to be normal in the *rhd6* mutant, because the distinctive cytoplasmic characteristics between H- and N-positioned cells are the same as in the wild type; apparently, only the hair-inducing machinery is impaired (Masucci and Schiefelbein, 1996). We found that the *rhd6* mutation inhibited the expression of both *AtEXP7* and *AtEXP18* almost completely (Figures 2K, 3, 8, and 10), suggesting that the molecular function of RHD6 is to regulate gene expression in the root hair cell either as a transcriptional regulator or as its upstream component. RHD6 may regulate the expression of the hair cell genes necessary for hair initiation, such as those involved in cytoskeletal dynamics, localized secretion, wall loosening, and wall synthesis. However, RHD6 probably does not target *AtEXP7* and *AtEXP18* directly, because expansin gene expression in *rhd6* can be restored by hormonal and environmental treatments. Our promoter analyses of the two expansin genes showed that ethylene, auxin, and root separation signals require the same promoter elements that control cell specificity (Figure 11). Thus, we propose that the signals from the developmental and environmental/hormonal pathways are merged at or before the transcription regulators that direct the hair cell specificity of the expansin genes. Identification and characterization of these transcription regulators will be important for understanding the mechanism of pattern formation in the root epidermis.

METHODS

Plant Materials

Arabidopsis thaliana was the model plant in this study. Unless indicated otherwise, the wild type was the Columbia ecotype. The mutant seeds of *ttg-1* (CS89), *gl2-1* (CS65), *eto2* (CS8059), *etr1-1* (CS237), *ers2-1* (CS8854), *ein4* (CS8053), *ctr1-1* (CS8057), and *ein2-1* (CS3071) were obtained from the ABRC (Columbus, OH). *rhd6* seeds were obtained from J.W. Schiefelbein (University of Michigan, Ann Arbor, MI). Seeds of the gain-of-function mutants *etr2* and *ers1* (Hua et al., 1995) were obtained from J. Hua (Cornell University, Ithaca, NY), and seeds of the loss-of-function mutant *etr1-7* were from E. Schaller (University of New Hampshire, Durham, NH). The seeds were sowed on agar plates including 4.3 g/L Murashige and Skoog (1962) nutrient mix (Sigma), 1% Suc, 0.5 g/L Mes, pH 5.7, with KOH, and 0.8% phytagar. After vernalization for 3 days, the seeds were germinated at 23°C under continuous light. For pharmacological experiments, 3-day-old seedlings were transferred to new plates containing growth regulators or antagonists and incubated for 1 additional day, after which root hairs and reporter gene expression patterns were examined. Transformed plants were selected on hygromycin-containing plates (10 μg/mL).

RNA Gel Blot Analyses

Total RNA preparation and RNA gel blot analyses were conducted as described previously (Cho and Kende, 1997). Gene-specific probes for *AtEXP7* and *AtEXP18* were generated from 3′ untranslated regions of the genes. To confirm equal amounts of RNA loading, the membranes were rehybridized with the Arabidopsis actin2 probe. Transcript levels were quantified from autoradiographs using Adobe Photoshop (Adobe Systems, San Jose, CA) as described previously (Cho and Cosgrove, 2000).

Reporter Gene Constructs

For the reporter gene constructs, the *AtEXP7* promoter region (between −1380 and +48 bp relative to the predicted transcription initiation site) from Arabidopsis BAC F5O11 was inserted into HindIII-XbaI sites of the binary vector pGPTV-HYG (Becker et al., 1992), which resulted in the *AtEXP7* promoter::*uidA* (β-glucuronidase [*GUS*]) construct. For the *AtEXP7* promoter::green fluorescent protein (*GFP*) construct, the *uidA* gene was replaced with the gene for GFP. The coding region of GFP was obtained from the pEGFP vector (Clontech, Palo Alto, CA) by PCR using primers 5′-AGTTGGAGCTCTCGAGTCGC-3′ (with the SacI site) and 5′-ATCCCCGGGTACCGGTC-3′ (with the SmaI site). This fragment of the GFP coding region replaced the *uidA* gene between the SacI and SmaI sites of the *AtEXP7* promoter::*GUS* construct. For the *AtEXP7* promoter::*AtEXP7-GFP* construct, in which the *AtEXP7* promoter directs the expression of the AtEXP7-GFP fusion protein, the coding region of *AtEXP7* was amplified from the genomic *AtEXP7* clone by PCR using primers 5′-CCTAAGAATCTAGAAAAAGAGGCTAGAATG-3′ (with the XbaI site) and 5′-AAAAGCCCGGGTAACACGGAAATTAGC-3′ (with the SmaI site). This fragment was inserted into XbaI-SmaI sites of the *AtEXP7* promoter::*GFP* construct. All of the constructs were confirmed by DNA sequencing. The constructs were introduced into Arabidopsis plants by *Agrobacterium tumefaciens* strain C58C1 (pMP90) using the vacuum infiltration method (Bechtold and Pelletier, 1998).

Detection of Reporters

GUS staining was performed as described previously (Cho and Cosgrove, 2000). For the detection of GFP, fluorescence from the seedling root was observed with a confocal laser scanning microscope (LSM-410; Carl Zeiss, Jena, Germany). For the cross-sectional view, 1- to 2-mm root sections were made after embedding the root in 1% agarose. To outline the cell boundary in some samples, the root was stained with propidium iodide (10 μg/mL). Green fluorescence was detected by excitation at 488 nm and emission at 543 nm. Red fluorescence from propidium iodide was detected by excitation at 568 nm and emission at 617 nm. Fluorescence images of the separate channels were digitized with LSM software version 3.5 (New Freedom, PA) and merged using Adobe Photoshop. The false red and green colors were adopted for propidium iodide and GFP fluorescence, respectively.

Observation of Root Hair Number and Length

The number of root hairs was determined using a differential interference contrast microscope according to Masucci and Schiefelbein (1996) with some modifications. For each seedling root, 5 consecutive epidermal cells from the same cell file were observed, and a total of 20 cells from two hair cell files and the adjacent two nonhair cell files were counted. Seven to 13 roots (for a total of 140 to 260 cells) per treatment or genotype were scored. Any protrusion was scored as the presence of the root hair, regardless of the length. In the root separated from the agar medium, total root hairs from the separated region were counted. For root separation, the agar medium immediately below the root tip was cut out, and the root was left to grow to the air. Root hair length was measured using a stereomicroscope when the root hair reached the maximum length. Seven root hairs per plant and five plants per genotype or treatment (for a total of 35 root hairs) were scored.

Treatment of 1-Methylcyclopropene

SmartFresh (0.14% 1-methylcyclopropene [1-MCP]) was obtained from H. Warner at Rohm and Haas (Spring House, PA). 1-MCP gas was produced by mixing the powder with water in a tightly sealed container according to the manufacturer's protocol. The gas was administered to the seedlings so that the final concentration was 1 or 10 μL/L in the container.

Promoter Analyses

The mutated *AtEXP7* promoters with 5′ deletions were prepared by PCR using the same reverse primer (5′-GGACCCATTCTAGACTCTTT-3′, containing the XbaI site) from the 3′ end (+48 bp) and the forward primers (containing the HindIII site) from the various 5′ ends, as indicated in Figure 8A, with the Arabidopsis BAC F5O11 clone as a template. Deletion of the *AtEXP18* promoter was performed similarly by PCR using a reverse primer (5′-TTTACTCTAGATTCTTGAGGGCGCCT-3′, containing the XbaI site) from the 3′ end (+42 bp) and the forward primers (containing the HindIII site) from the 5′ ends, as shown in Figure 10A, with the Arabidopsis BAC F16P17 clone as a template.

Substitution mutagenesis of the proximal region (−134 to −70 bp) of the *AtEXP7* promoter, designated E7M1~E7M7 in Figure 9A, was performed using the "megaprimer PCR" method (Barik, 1995). The megaprimers were amplified using the forward primer 5′-TAGTTAAGCTTTGGAAACGTAA-3′ (located at −386 bp and containing the HindIII site) and the mutagenized reverse primers from the regions indicated in Figure 9A. The second PCR was performed with these megaprimers and the same reverse primer that was used for the deletion analysis.

The gain-of-function promoters of *AtEXP7* were made by associating diverse lengths of proximal promoter parts with the minimal 35S promoter of *Cauliflower mosaic virus* (mp35S). The mp35S region (−64 35S promoter; Eyal et al., 1995) was produced by PCR using the forward primer 5′-AAGGGTCTAGACACAATCCCACTA-3′ (containing the XbaI site) and the reverse primer 5′-GACCACCCGGGGATCCACTA-3′ (containing the SmaI site) from the pBI121 vector (Clontech). This PCR product was inserted between the XbaI and SmaI sites of pGPTV-HYG so that mp35S was followed by the GFP reporter gene. The gain-of-function *AtEXP7* promoters E7G1 to E7G3, as shown in Figure 9A, were prepared by complementing the sense and antisense oligonucleotides, which contained HindIII and XbaI sites at their 5′ and 3′ flanking regions, respectively. The E7G4 gain-of-function promoter was produced by PCR amplification of the region between −134 and −46 bp. E7G4M6 and E7G4M7 were

made from the same region from which E7G4 was made except that PCR was performed with the E7M6 and E7M7 constructs as templates, respectively.

The truncated or substituted promoter fragments were inserted between the HindIII and XbaI sites of the pGPTV-HYG vector so that the promoters were followed by the GFP reporter gene, and the gain-of-function promoters were inserted between the HindIII and XbaI sites of the pGPTV-HYG vector containing mp35S::GFP. The constructs were introduced into Arabidopsis plants (either the wild type or *rhd6*) using Agrobacterium as described above. The first generation of transformants (T1, 9 to 62 independent lines per construct) was used to quantify the relative expression levels of GFP in the root. After selection for 5 days on hygromycin-containing plates, the transformants were transferred to new plates without effectors for the wild-type background or with 1-aminocyclopropane-1-carboxylic acid (5 μM), indole-3-acetic acid (30 nM), or separation of the root from the agar medium for the *rhd6* mutant, and GFP expression was observed 1 day after the treatments.

To evaluate the promoter activity (GFP expression), fluorescence images of roots were taken digitally using a confocal laser scanning microscope. Relative brightness from the digital images was quantified using the histogram function in Adobe Photoshop. For the histogram analysis, a rectangular marquee (4 × 3 of the root diameter) was located around the root, where GFP fluorescence is maximal, and the mean value was read from the histogram window. The final relative brightness was calculated by subtracting the background values.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The accession numbers for the genes described in this article are AC025416 (*AtEXP7*), AC011000 (*AtEXP18*), and U41998 (*AtACT2*).

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